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Effect of DOPA-Loading on Glutathione-dependent 5-S-Cysteinyldopa Genesis in Melanoma Cells *in Vitro*

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The effect of dopa, cysteine, and glutathione on 5-S-cysteinyldopa (5-S-CD) genesis in melanoma cells cultured in normal and tyrosine- and cysteine-free media has been studied. In normal media only melanotic melanoma cells have been found to secrete 5-S-CD into the medium. In the presence of dopa and cysteine, both, media incubated with and without cells have been found to produce 5-S-CD. In the presence of dopa and glutathione, however, cell-free media did not show the presence of 5-S-CD. In contrast melanoma cell-cultured media has been found to contain large quantities of this amino acid. The optimum condition for maximum production of 5-S-CD via glutathione-dependent pathway has been found to be at the dopa concentration of 10^{-5} M when glutathione is present at the concentration of 10^{-5} M in the culture medium. Thus dopa concentration with regards to glutathione is 1:1 on the molar basis which is twice the dopa concentration required in *in vitro* noncellular tyrosinase system. It is suggested that higher dopa requirement in our melanoma cell culture system reflects the co-existence of eu- and pheomelanin synthesis taking place according to their genetically predetermined proportions.

Recent advances in the chemistry of melanogenesis have revealed molecular mechanisms governing the fate of dopaquinone towards formation of eumelanin and the lately much clarified pheomelanin via cysteinyldopas (Cys-dopas) [1]. Of these Cys-dopas, 5-S-cysteinyldopa (5-S-CD) has been shown to be the major intermediate by Prota and his associates [2]. Parallel to these chemical investigations this amino acid has been shown to be present in substantial amounts in the melanomas of Caucasians [3] and Negroes [4]. Rorsman and his group further showed that 5-S-CD is excreted in large quantities in the urine of melanoma patients [5]. In 1979, we reported that melanomas and the urine of melanoma patients of Mongoloid origin also contain substantial amounts of 5-S-CD [6]. In contrast nonpigment cell tumors were found to be lacking in this amino acid.

However, both animal and human melanomas manifest significant variations in their 5-S-CD content. Similarly, healthy normal subjects and melanoma patients show significant individual and seasonal variations in the excretion of this amino acid [7,8]. These variations may be due to the complex metabolic factors involved in the maintenance of a whole biological being as well as melanoma cells themselves. In this paper, our studies on the regulatory factors—substrates or chemicals involved in 5-S-CD genesis in melanoma cells cultured away from such intricate biological influences are reported.

MATERIALS AND METHODS

The following cell lines maintained in our laboratories were used for the study:

1. B-16 CB, a mouse melanotic melanoma cell line,
2. B-16 C_2W, amelanotic derivative of B-16 CB,
3. Greene's melanotic melanoma of the hamster, and
4. NH2KO, fibroblasts from normal skin.

Normal Media

The cells were cultured in Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS) for 4–7 days at 37°C under 5% CO_2. The medium was then collected in the presence of 0.2 ml 1% sodium metabisulfite and 0.2 ml acetic acid and assayed for 5-S-CD [9]. The cells were simultaneously trypsinized and counted.

Experimental Media

Eagle's MEM, along with other constituents also contains 2 amino acids, tyrosine (36 mg/l) and cystine (24 mg/l) that are involved in the synthesis of melanin. The dopa-loading experiments were therefore carried out in tyrosine and cystine free media. The exact composition of this experimental media is the same as Eagle's MEM except for the absence of tyrosine and cystine. Also in these experiments the concentration of FCS was reduced to only 1% in order to minimise unknown factors affecting 5-S-CD genesis. The cells originally cultured in MEM + 10% FCS for 3–4 days were rinsed and recultured in duplicate sets in experimental media in the presence of known quantities of dopa and glutathione or cysteine. After an incubation period of 20 hr the medium was collected and the cells counted as mentioned above.

a. Glutathione-Dependent 5-S-CD Genesis

Preliminary experiments had revealed that dopa 10^{-5} M and glutathione 10^{-5} M appear to be appropriate conditions for 5-S-CD genesis in cultured melanoma cells. Therefore in dopa-loading experiments the glutathione concentrations were kept constant at 10^{-5} M while dopa concentrations were varied from 0 to 10^{-4} M. Experiments in reversed conditions, i.e., keeping dopa constant at 10^{-5} M and changing glutathione concentrations were also carried out.

b. Cysteine-Dependent 5-S-CD Genesis

The experiments were similar to those mentioned in (a) except that glutathione was replaced by cysteine.

c. Controls

In every case media similarly incubated, but without cells served as controls.

Extraction and Assay Procedures

5-S-CD from the medium was eluted after $A1_20_3$ adsorption according to the technique of Anton and Sayre [10] and assayed by the spectrofluorometric method of Rorsman, Rosengren, and Rosengren [11]. Pure 5-S-CD for standardisation was generously provided by Prof. H. Rorsman, Lund, Sweden.

RESULTS

Normal Media

Table I gives the levels of 5-S-CD found in the medium of different cell lines cultured in MEM + 10% FCS. The medium

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Abbreviations:
Cys-dopa: cysteinyldopas
5-S-CD: 5-S-cysteinyldopa
FCS: Fetal calf serum
MEM: Eagle's minimum essential medium

in which the melanotic melanoma cells were cultured were found to contain various amounts of 5-S-CD. In contrast, the medium in which amelanotic melanoma and nonpigment cells were cultured did not contain 5-S-CD. The results indicate that only melanin producing cells produce this amino acid.

Experimental Media

Dopa, cysteine, and glutathione when added individually did not raise the amount of 5-S-CD in the medium of cultured melanoma cells. However, when dopa and either of the thiols were added together, significant differences in their responses to 5-S-CD production were observed.

a. Dopa and glutathione: In the presence of these 2 compounds, melanoma cells were found to secrete large quantities of 5-S-CD into their medium. Media incubated similarly with dopa and glutathione, but without cells did not show the presence of this amino acid. Fig 1 (*a*) shows dopa concentration dependent 5-S-CD production in the presence of 10^{-5} M glutathione and Fig 1 (*b*) shows glutathione dose response in the presence of 10^{-5} M dopa by B-16 CB cells. At the concentrations used, dopa or glutathione was not found to be toxic as revealed by cell counts.

b. Dopa and cysteine: When dopa and cysteine were used in our cell culture medium, both cell-free and cell-cultured media were found to produce large quantities of 5-S-CD. Table II gives the details of such 5-S-CD production in tyrosine- and cystine-free Eagle's MEM in the presence of dopa and cystine.

Cell Number Dependent 5-S-CD Production

At the concentration 10^{-5} M of dopa and 10^{-5} M glutathione the amount of 5-S-CD secreted into the medium has been found to depend on the number of melanoma cells seeded into the medium (Fig 2). These results indicate that in the presence of dopa and glutathione, production and secretion of 5-S-CD are carried out by viable melanoma cells.

TABLE I. *The levels of 5-S-cysteinyldopa released by various types of cells cultured in Eagle's MEM + 10% FCS*[a]

Cell line	μg 5-S-CD/total medium	No. of days of incubation
B-16 CB	0.86	7
Greene's Mm	1.18	4
B-16 C_2W	0.0	7
NH2KO	0.0	7

[a] MEM contains 36 mg of tyrosine and 24 mg of cystine per litre. Dopa and glutathione are not present in normal MEM.

DISCUSSION

Despite much clarification of 5-S-CD genesis in defined chemical systems (1,2 and Fig 3) and on urinary excretion of 5-S-CD [3–6], there has been little work reported about the substances modulating 5-S-CD genesis in melanogenically active pigment cell systems.

TABLE II. *Comparison between melanoma cell-containing and cell-free 5-S-cysteinyldopa production in the presence of dopa and cysteine*[a]

Dopa, μg	Cysteine, μg	μg 5-S-CD formed	
		Cell-containing	Cell-free
10	2.5	1.34	0.0
10	5.0	1.34	1.28
10	10.0	7.00	5.67
10	20.0	3.36	3.17
10	40.0	1.73	1.67

[a] The medium used is tyrosine and cystine free Eagle's MEM + 1% FCS. The incubation time is 5 min.

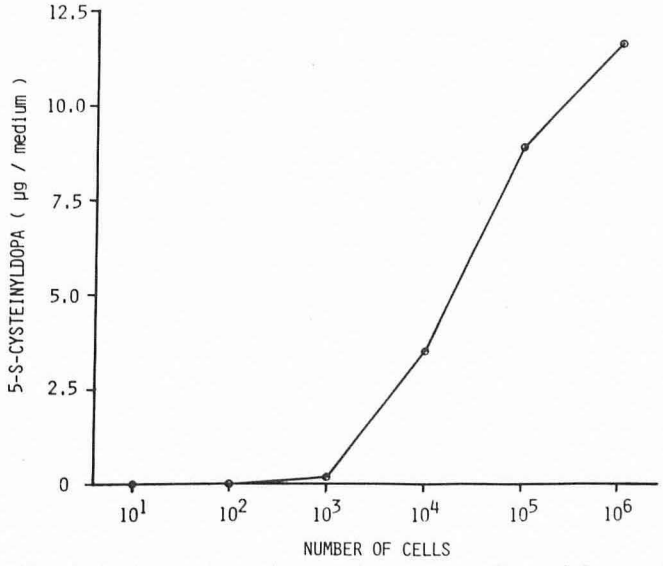

FIG 2. In the presence of equimolar concentrations of dopa and glutathione the amount of 5-S-CD released into the medium has been found to be proportional to the number of cells seeded into the flask. The culture conditions were identical to those mentioned in Fig 1.

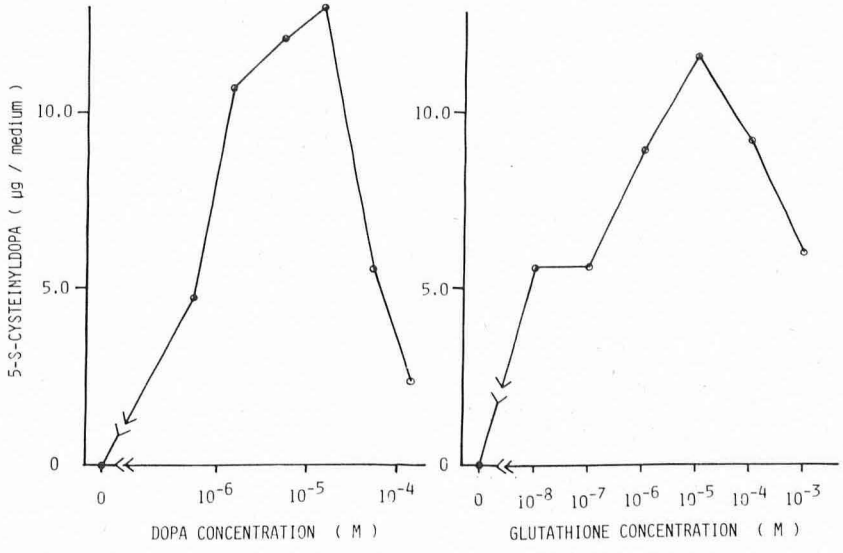

FIG 1. The levels of 5-S-CD secreted into the medium (6 ml) by B-16 CB melanoma cells in response to dopa and glutathione concentrations. The cells were cultured in tyrosine- and cystine-free Eagle's MEM supplemented with only 1% FCS. The maximum amount of 5-S-CD production has been found to occur when equimolar concentrations of dopa and glutathione are present. M.W. of 5-S-CD; 337. 39.

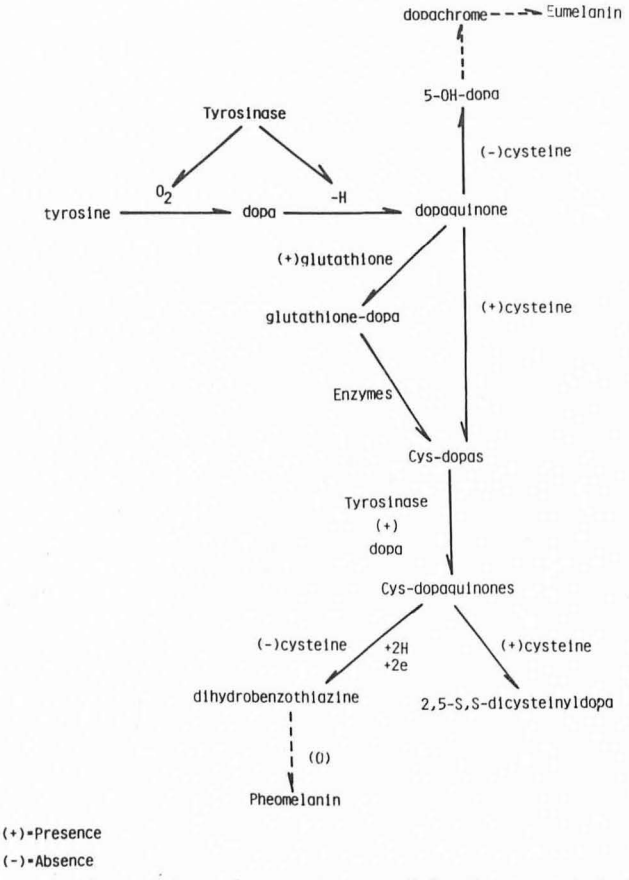

FIG 3. Current views of dopaquinone- and Cys-dopa-genesis during eu- and pheomelanin formation revealed by noncellular and cellular tyrosinase *in vitro* systems.

In contrast to *in vitro* chemical systems of Prota [2], where tyrosinase was necessary to produce 5-S-CD from dopa and cysteine at pH 6.8, in our culture medium this amino acid has been found to be formed from dopa and cysteine even in the absence of tyrosinase and tyrosinase-containing melanoma cells (Table II). This would indicate that 5-S-CD can be nonenzymatically formed by the nucleophillic addition of cysteine to dopaquinone which is produced by the auto-oxidation of dopa at the pH required to maintain the cells.

A similar *in vitro* chemical system has been used by Rorsman, Rosengren, and Rosengren to produce glutatione-dopa and not cysteinyldopa from dopa and glutathione [12]. Differing from these noncellular tyrosinase systems however, in our viable pigment cell system, the addition of dopa and glutathione leads to the secretion of large amounts of 5-S-CD (Fig 1 and 2). It is therefore apparent that glutathione-dopas formed by the tyrosinase catalyzed reaction are further converted to Cys-dopas within melanoma cells *in vitro*. This is in agreement with Rorsman and his group's recent findings that homogenates of melanoma as well as kidney can bring about this metabolic conversion [13].

In defined chemical systems, optimum conditions for the 5-S-CD yield is reported to be at the initial concentration ratio of dopa:thiols = 1:2 on the molar basis [2]. In contrast to this, in our present *in vitro* melanoma cell experimental condition, optimum yield of 5-S-CD has been found to be when the initial molar ratio of dopa:glutathione is 1:1. This finding of higher concentrations of dopa requirement for producing optimum conditions for 5-S-CD yield in our cell culture system seems to indicate the possibility that, both eumelanogenesis involving the conversion of dopa to dopa-chrome and pheomelanogenesis involving the conversion of dopa to Cys-dopa are taking place simultaneously within these cells (Fig 3).

When greater than 1:1 molar ratio of dopa is loaded into the glutathione-dependent 5-S-CD production in the present melanoma cell cultured system, an acute drop in 5-S-CD yield has been found (Fig 1). In a recent study using tyrosine instead of dopa Rorsman, Rosengren and Agrup [14] have shown that when dopa is still being formed from tyrosine by tyrosinase after the conversion of all cysteine to Cys-dopa, 5-S-CD rapidly disappears being converted to monocysteinyldopaquinones. The acute drop in 5-S-CD levels observed in our tissue culture medium when dopa:thiol ratio is 2:1 suggests that a similar mechanism is also involved within cells.

This finding led us to investigate further the dynamics of glutathione-dependent 5-S-CD genesis in viable melanoma cells by modulating the levels of exogenous glutathione. As shown in Fig 1 (*b*), it has been found that when glutathione concentrations are more than 1:1 with regards to the dopa concentration there is a fall in the levels of 5-S-CD. Agrup et al [13] reported that at the concentration of 3×10^{-2} M, glutathione inhibits completely the conversion of glutathione-dopa to Cys-dopa by dialyzed homogenates of melanoma and kidney *in vitro*. It has therefore become apparent that the enzymes such as γ-glutamyl transferase involved in the catabolism of glutathione are responsible for the splitting of glutathione-dopa to Cys-dopa also in viable melanoma cells.

The above evidence would indicate that 5-S-CD formation observed in *in vitro* bio-mimetic chemical systems is representing the metabolic pathways actually occuring within viable pigment cells. Further, the results also suggest that the regulatory mechanisms involved in the production of eu- and pheomelanin according to their genetically predetermined proportions and the switching over from eu- to pheomelanogenesis that occurs within hair bulb melanocytes of agouti mice may largely be due to the availability of free sulfydryl compounds within the melanocytes. The mechanisms for these however, require further investigations.

REFERENCES

1. Prota G: Recent advances in the chemistry of melanogenesis in mammals. J Invest Dermatol 15:122–127, 1980
2. Ito S, Prota G: A facile one-step synthesis of cysteinyldopas using mushroom tyrosinase. Experientia 33:1118–1119, 1977
3. Agrup G, Falck B, Jacobsson S, Rorsman H, Rosengren A-M, Rosengren E: 5-S-cysteinyldopa in melanomas of caucasians. Acta Derma Vernereol (Stockh) 54:21–22, 1974
4. Vogel CL, Dhru DH, Rorsman H, Rosengren A-M, Rosengren E: Dopa and 5-S-cysteinyldopa in melanomas of caucasians. Acta Derma Venereol (Stockh) 54:21–22, 1974
5. Agrup G, Agrup P, Andersson T, Falck B, Hansson J-A, Jacobsson S, Rorsman H, Rosengren A-M, Rosengren E: Urinary excretion of 5-S-cysteinyldopa in patients with primary melanoma or melanoma metastasis. Acta Derm Venereol (Stockh) 55:337–341, 1975
6. Mojamdar M, Ichihashi M, Mishima Y: Detection and quantitation of 5-S-cysteinyldopa in melanotic and amelanotic melanoma in comparison with nonpigment cell tumors and it's urinary excretion. J Dermatol (Tokyo) 6:379–382, 1979
7. Ichihashi M, Mojamdar M, Mishima Y: Experimental and clinical investigation on the dynamics of 5-S-cysteinyldopa in malignant melanoma in vivo: Production, excretion and regulatory factors. Proceedings of the XIth International Pigment Cell Conference, Pigment Cell 6:579–586, 1982
8. Rorsman H, Agrup P, Falck B, Rosengren A-M, Rosengren E: Exposure to sunlight and urinary excretion of 5-S-cysteinyldopa, Pigment cell, vol 2. Edited by V Riley. Basel, S. Karger, 1976, pp 284–289
9. Aubert C, Rosengren E, Rorsman H, Rougé F: Differentiation of melanocytes in cultures of primary malignant melanoma indicated by 5-S-cysteinyldopa formation. J Natl Can Inst 55:1327–1328, 1975
10. Anton AH, Sayre DR: The distribution of dopamine and dopa in various animals and a method for their determination in diverse biological materials. J Pharmacol Exp Ther 145:326–336, 1964
11. Rorsman H, Rosengren A-M, Rosengren E: A sensitive method for the determination of 5-S-cysteinyldopa. Acta Derm Venereol (Stockh) 53:248–250, 1973
12. Rorsman H, Rosengren A-M, Rosengren E: Fluorometry of catechol thioethers. Acta Derm Venereol (Stockh) 52:353–356, 1972
13. Agrup G, Falck B, Kennedy B-M, Rorsman H, Rosengren A-M, Rosengren E: Formation of cysteinyldopa from glutathionedopa in melanoma. Acta Derm Venereol (Stockh) 55:1–3, 1975
14. Rorsman H, Hansson C, Rosengren E, Agrup P: Oxidation of dopaquinone addition products. Proceedings of the XIth International Pigment Cell Conference, in press